Virulence- Addendum

Haptoglobin-Hemoglobin Receptor Independent Killing of African Trypanosomes by Human Serum and Trypanosome Lytic Factors

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ABSTRACT

The haptoglobin-hemoglobin receptor (HpHbR) of African trypanosomes plays a critical role in human innate immunity against these parasites. Localized to the flagellar pocket of the veterinary pathogen *Trypanosoma brucei brucei* this receptor binds Trypanosome Lytic Factor-1 (TLF-1), a subclass of human high-density lipoprotein (HDL) facilitating endocytosis, lysosomal trafficking and subsequent killing. Recently, we found that group 1 *Trypanosoma brucei gambiense,* does not express a functional HpHbR. We now show that loss of the *Tbb*HpHbR reduces the susceptibility of *T. b. brucei* to human serum and TLF-1 by 100 and 10,000 fold respectively. The relatively high concentrations of human serum and TLF-1 needed to kill trypanosomes lacking the HpHbR indicates that high affinity *Tbb*HpHbR binding enhances the cytotoxicity, however in the absence of *Tbb*HpHbR other receptors or fluid phase endocytosis are sufficient to provide some level of susceptibility. Human serum contains a second innate immune factor, TLF-2, that has been suggested to kill trypanosomes independently of the *Tbb*HpHbR. We found that *T. b. brucei* killing by TLF-2 was reduced in *Tbb*HpHbR deficient cells but to a lesser extent than TLF-1. This suggests that both TLF-1 and TLF-2 can be taken up via the *Tbb*HpHbR but that alternative pathways exist for the uptake of these toxins. Together the findings reported here extend our previously published studies (Kieft *et al.,* 2010) and suggest that group 1 *T. b. gambiense* has evolved multiple mechanisms to avoid killing by trypanolytic human serum factors.

INTRODUCTION

African trypanosomes are eukaryotic pathogens that cause important human and animal diseases. These parasites have evolved a variety of mechanisms to escape innate and acquired immunity including the use of the variant surface glycoprotein (VSG) coat to cover the plasma membrane of the parasite providing a barrier against attack by complement[1]. The VSG coat also serves as the molecular decoy during antigenic variation presenting an ever-changing target to the adaptive immune system of the mammal, thus allowing the parasites to evade antibody mediated killing [2]. The subspecies of trypanosomes that infect humans face the additional challenge of encountering a unique innate defense mechanism mediated by two related serum proteins complexes. In the circulation of humans, TLF-1 is a minor subclass of HDL containing apolipoprotein A-1 (apoA-1), the defining protein of all HDLs, and two primate specific proteins, apolipoprotein L-1 (apoL-1) and haptoglobin-related protein (Hpr) [3,4,5,6,7]. In addition to these apolipoproteins, Hpr binds free hemoglobin (Hb) in the circulation, which is likely released from erythrocytes during early infection[8]. The heterodimeric Hpr/Hb complex is proposed to be bifunctional, serving both as the ligand for the *T. b. brucei* HpHbR [9,10] and directly contributing to high specific activity killing by catalyzing the peroxidation of lysosomal membrane lipids[6,7,11]. The other primate specific apolipoprotein in TLF-1, apoL-1, is also directly involved in *T. b. brucei* killing[5,12,13]. An ion channel forming protein, apoL-1 undergoes conformation changes at lysosomal pH and can integrate into membranes[5,12,14]. The combined action of Hpr/Hb and apoL-1 results in the osmotic lysis of the parasite[15,16]. The other

trypanolytic serum complex is called TLF-2 and, while largely devoid of lipids it contains Hpr and apoA-1[17] and apoL-1 (this paper) suggesting that these complexes share a common origin and perhaps have a similar mechanism of trypanosome killing[18].

The two subspecies of human sleeping sickness trypanosomes have evolved distinct mechanisms to survive in the human host. *Trypanosoma brucei rhodesiense* produces the serum resistance associated (SRA) protein that binds and inhibits TLF-1[19,20,21]. SRA, an intracellular protein largely found in endosomes, co-localizes with TLF-1 in early endosomes and trafficks to the lysosome[22]. Thus, *T. b. rhodesiense* survives in humans largely because it is able to produce an antidote to TLF-1. While untested, it is likely that TLF-2 is inhibited by SRA by the same mechanism since both serum complexes contain apoL-1. In contrast, we recently showed that group 1 *T. b. gambiense* does not bind or take up TLF-1 suggesting that these cells have evolved a different mechanism to avoid the cytotoxicity of TLF-1 [23]. The underlying basis for reduced TLF-1 uptake is two fold. First, *Tbg*HpHpR is expressed at very low levels by group 1 *T. b. gambiense* and second, *TbgHpHbR* contains a number of point mutations within the coding sequence, that render the receptor non-functional[23]. The combination of mutations to the *TbgHpHbR* and reduced expression abolished TLF-1 binding and uptake resulting in resistance to TLF-1. Thus, in contrast to *T. b. rhodesiense*, it appears the mechanism of group 1 *T. b. gambiense* resistance to TLF-1 involves reduced uptake and avoidance of the toxin. To date no evidence for an inhibitory protein with SRA-like characteristics has been described in *T. b. gambiense*. In this short addendum to the Kieft *et al.* (2010) paper

we now show that while the *Tbb*HpHbR enhances susceptibility to human serum, TLF-2 and TLF-1 other receptors or fluid phase endocytosis also contribute to trypanosome killing. Further, our results suggest that the resistance of group 1 *T. b. gambiense* to human serum and TLF involves other mechanisms beyond the simple loss of a single receptor.

MATERIAL & METHODS

TLF-1 and TLF-2 Purification. Total serum was obtained from a healthy human donor. As previously described, two sequential flotations on sodium bromide gradients (ρ=1.063 and 1.26 g/ml) resulted in an HDL-rich fraction (TLF-1; top third of the gradient) and a lipoprotein deficient fraction (TLF-2; bottom third of the gradient)[4]. The TLF-1 fraction was passed over an anti-IgM column (Sigma, A9935). The unbound material was then passed over an anti-Hpr column, washed with PBSE (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 3 mM EDTA) and bound protein was eluted in 100 mM glycine (pH 2.5) and neutralized with 1 M Tris (pH 7.5). The TLF-2 fraction was passed over an anti-Hpr column and washed with PBSE. Bound protein was eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris (pH 7.5) and immediately added to an anti-IgM column and washed with PBSE. Bound protein was eluted in 100 mM glycine (pH 2.5), neutralized with 1 M Tris (pH 7.5). All protein samples were aliquoted and stored at -80^{0}C.

Size Exclusion Chromatography and Western Blot Analysis. Size exclusion chromatography was performed on a 1 X PBSE equilibrated Superose 6 10/300 GL column (GE Healthcare). Individual protein standards were used to estimate the molecular weights of TLF-1 and TLF-2. Samples of TLF-1 and TLF-2 from immuno-affinity purification (70 μg) were run on the Superose 6 column at a flow rate of 0.5 ml/min. Fractions were collected (0.5 ml), proteins concentrated six fold with microspin S-300HR columns (GE, 27513001) and the distribution of Hpr, apoL-1 and IgM determined by SDS-PAGE, silver staining and western blot analysis. Characterization of antibodies against Hpr and apoL-1 has previously been described[7]. Anti-IgM was purchased from Sigma and used according to the manufacturers recommendation (Sigma, I0759).

RESULTS

TLF-1 resistant T. b. brucei. During the course of our studies on the mechanism of TLF-1 resistance in group 1 *T. b. gambiense* we developed a laboratory model for TLF-1 resistance using well-characterized clonal cell lines of *T. b. brucei* that had been selected for resistance to human HDLs[23,24]. We isolated TLF-1 resistant (R) or susceptible (S) *T. b. brucei* lines expressing either the VSG800 or VSG060[23]. The *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} lines showed reduced uptake of TLF-1 relative to the TLF-1 susceptible parental *T. b. brucei* 427-221^{S} cells and TLF-1 susceptible cells expressing either VSG800 or VSG060. In addition, we showed that the expression of *Tbb*HpHbR mRNA was reduced approximately 20-fold in resistant cells[23]. These findings led us to examine group 1 *T. b. gambiense* where we found

that not only was expression of the *Tbg*HpHbR mRNA reduced but that mutations to the gene abolished function [23].

Purification and characterization of TLF-1 and TLF-2. In order to determine whether loss of *Tbb*HpHbR was sufficient to provide complete protection from human serum, TLF-1 and TLF-2 activity we developed a purification protocol exploiting physical and compositional differences in these human serum innate immune complexes (Figure 1). Freshly collected human plasma was initially separated by density gradient ultracentrifugation to produce HDL-enriched (ρ 1.063 – 1.26 g/ml) and lipoprotein-deficient fractions (ρ <1.063 g/ml) that were used as the starting materials for TLF-1 and TLF-2 purification respectively. During the purification of TLF-1 small amounts of contaminating TLF-2 were removed from the HDL-containing fraction by absorption with anti-IgM. TLF-1 was subsequently bound to anti-Hpr resin, washed extensively at neutral pH to remove human HDLs lacking Hpr and eluted at low pH. TLF-2 was purified from the lipoprotein-deficient serum by sequential affinity chromatography with anti-Hpr followed by binding and elution from an anti-IgM column. The purity of TLF-1 and TLF-2 was evaluated by size exclusion chromatography on Superose 6 and western blot analysis with anti-Hpr, apoL-1 and IgM (Figure 1A and B). Based on size exclusion chromatography TLF-1 and TLF-2 have estimated relative sizes of 576KDa and 1.6MDa respectively[17]. Superose 6 chromatography of purified TLF-1 and TLF-2 revealed somewhat dispersed distributions consistent with particle heterogeneity but there was minimal overlap of the TLF-1 and TLF-2 absorbance peaks at 280nM (Figure 1A). Western blot analysis revealed no contaminating TLF-2 in our purified TLF-1

preparations based on the lack of anti-IgM reactive material on western blots (Figure 1B; data not shown). TLF-2 preparations were highly enriched in particles containing Hpr, apoL-1 and IgM, however, these preparations also contained small amounts of IgM deficient complexes with an elution time (~28 min) from the Superose 6 column consistent with TLF-1. Based on the distribution of the Hpr dimer and IgM across the size exclusion fractions we estimate the amount of contaminating TLF-1 in these preparations to be ~10%.

Susceptibility of T. b. brucei to human serum, TLF-1 and TLF-2. Our previous studies compared the short-term killing of trypanosomes to TLF-1[23]. Here we have reexamined the susceptibility of these *T. b. brucei* lines using a long-term growth assay (Figure 2). Consistent with previous studies, the parental *T. b. brucei* 427-221^{S}, *T. b. brucei* 427-800^{S}, and *T. b. brucei* 427-060^{S} were highly susceptible to TLF-1 with a calculated LG_{50} of 0.8-6 ng/ml. *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} were >10,000 fold more resistant to TLF-1 than wild type *T. b. brucei* suggesting the *Tbb*HbHbR is important in TLF-1 susceptibility. However, concentrations of >10 μg/ml overcame the *Tbb*HpHbR deficiency leading to reduced survival (Figure 2A). Since the concentration of TLF-1 needed to kill *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} is similar to that found in human serum it is likely that, *Tbb*HpHbR independent mechanisms of TLF-1 uptake play a significant role in trypanosome killing.

Based on our studies with both the group 1 *T. b. gambiense* and the TLF-1 resistant *T. b. brucei* lines we predicted that loss of a functional *Tbg*HpHbR played a critical

role in human infection by African trypanosomes[23]. The dramatic reduction in susceptibility to TLF-1 in the *Tbb*HpHbR deficient cell lines supports this prediction (Figure 2A). However, the possibility remained that human serum contained additional innate immune factors, such as TLF-2, that might not require the *Tbb*HpHbR. To test this possibility, we treated TLF-1 resistant and susceptible *T. b. brucei* lines with human serum (Figure 2B). We found that *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} were approximately 100 fold more resistant to human serum killing than either *T. b. brucei* 427-800^{S} or *T. b. brucei* 427-060^{S} (Figure 2B). Based on these results, we conclude that loss of *Tbb*HpHbR expression contributes to the overall resistance of these cells to human serum, however the level of resistance is much less than the high level of resistance seen for TLF-1 (10,000 fold). A possible interpretation of these findings is that other human serum components, such as TLF-2, are less dependent on *Tbb*HpHbR binding than TLF-1.

It has been proposed that TLF-2 can bind to *T. b. brucei* in the absence of the *Tbb*HpHbR[18]. We tested whether highly purified TLF-2 was able to kill *T. b. brucei* 427-800^{R} and *T. b. brucei* 427-060^{R} (Figure 2C). Similar to our findings with complete human serum, these *Tbb*HpHbR deficient cells were more resistant to TLF-2 relative to the wildtype, *Tbb*HpHbR expressing, cell lines. Thus, reduced expression of *Tbb*HpHbR expression caused a reduced susceptibility of TLF-2 killing suggesting that TLF-2 can bind to the *Tbb*HpHbR. However, the toxic concentration of TLF-2 is >10 fold less than TLF-1 indicating that *Tbb*HpHbR independent mechanisms may play a greater role in TLF-2 binding, uptake and killing.

DISCUSSION

In the studies presented here human serum, TLF-1 and TLF-2 susceptibility was examined in isogenic lines of *T. b. brucei* differing in *Tbb*HpHbR expression. Cells deficient in *Tbb*HpHbR expression were 10,000 fold more resistant to TLF-1 relative to wild type, susceptible cells. However, at concentrations of TLF-1 typically found in serum (>10μg/ml), both resistant and susceptible cell lines were killed. Human serum killing was also reduced approximately 100 fold in cell lines expressing reduced levels of *Tbb*HpHbR. However, significant killing was still observed at human serum concentrations above 100 μg/ml. Since the only difference in the susceptible and resistance cell lines is the levels of expression of *Tbb*HpHbR it seems likely that human serum contains a second trypanolytic activity that interacts with *T. b. brucei* independently of the *Tbb*HpHbR. Based on this interpretation of the human serum killing results we decided to investigate whether TLF-2 killing of *T. b. brucei* was independent on the level of expression of *Tbb*HpHbR. We found that TLF-2 killing was reduced 500-1000 fold in cell lines with reduced levels of *Tbb*HpHbR suggesting that TLF-2 also binds *Tbb*HpHbR. These results are in apparent contrast to previous studies on TLF-2 showing that TLF-2 killing was not inhibited by the addition of haptoglobin, an inhibitor of HpHb binding to the TbbHpHbR[17]. These results have been used subsequently to argue that TLF-2 does not bind the TbbHpHbR[10]. It is possible that our results are influenced by the small amount of contaminating TLF-1 in our TLF-2 preparations. Clearly, a detailed characterization of the TLF-2 binding, uptake and cellular location is needed.

Our results are consistent with previous findings indicating the importance of the *Tbb*HpHbR in TLF-1 killing but also suggest that other mechanisms of TLF-1 binding and uptake may contribute to trypanosome killing. The most likely pathways for *Tbb*HpHbR independent uptake of TLF-1 is either by fluid phase endocytosis or the trypanosome lipoprotein scavenger receptor[25,18]. The findings presented here further support the findings of others that TLF-2 killing is less dependent on the *Tbb*HpHbR than is TLF-1[17,10]. Finally, we propose that group 1 *T. b. gambiense* have evolved multiple mechanisms, including but not limited to the loss of a functional HpHbR, to avoid the cytotoxicity of the trypanosome lytic factors. We are currently exploring these mechanisms.

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REFERENCES

1. Pays, E The variant surface glycoprotein as a tool for adaptation in African trypanosomes. Microbes Infect. 2006; 8: 930-937.

2. Taylor, JE, Rudenko,G Switching trypanosome coats: what's in the wardrobe. Trends Genet 2006; 18:614-620.

3. Rifkin, MR Identification of the trypanocidal factor in normal human serum: high-density lipoprotein. Proc Natl Acad Sci USA 1978; 75: 3450-3454.

4. Hajduk, SL, Moore, DR, Vasudevacharya, J, Siqueira, H, Torri, AF, Tytler, EM, Esko, JD Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. J Biol Chem 1989; 264: 5210-5217.

5. Vanhamme, L, Paturiaux-Hanocq, F, Poelvoorde, P, Nolan, DP, Lins, L, Van Den Abbeele, J, Pays, A, Tebabi, P, Van Xong, H, Jacquet, A*, et al.* Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature 2003; 422: 83-87.

6. Smith, AB, Esko, JD, Hajduk, SL Killing of trypanosomes by human haptoglobin related protein. Science 1995; 268:284-286.

7. Shiflett, AM, Bishop, JR, Pahwa, A, Hajduk, SL Human high density lipoproteins are platforms for the assembly of multi-component innate immune complexes. J Biol Chem 2005; 280: 32578-32585.

8. Widener, J, Nielsen, MJ, Shiflett, A, Moestrup, S, Hajduk, S Hemoglobin is a co-factor of human trypanosome lytic factor. PLoS Pathogens 2007; 3: 1250-1261.

9. Drain, J, Bishop, R, Hajduk, SL Haptoglobin-related protein mediates trypanosome lytic factor binding to trypanosomes. J Biol Chem 2001; 276: 30254-30260.

10. Vanhollebeke, B, De Muylder, G, Nielsen, MJ, Pays, A, Tebabi, P, Dieu, M, Raes, M, Moestrup, SK, Pays, E A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 2008; 320: 677-81.

11. Bishop, JR, Shimamura, M, Hajduk, SL Insight into the mechanism of trypanosome lytic factor-1 killing of *Trypanosoma brucei brucei*. Mol Biochem Parasitol 2001; 118: 33-40.

12. Perez-Morga, D, Vanhollebeke, B, Paturiaux-Hanocq, F, Nolan, DP, Lins, L, Homble, F, Vanhamme, L, Tebabi, P, Pays, A, Poelvoorde, P, Jacquet, A, Brasseur,R, Pays, E Apolipoprotein L-I promotes trypanosome lysis by forming pores in lysosomal membranes. Science 2005; 309: 469-472.

13. Molina-Portela, MP, Samanovic, M, Raper, J Distinct roles of apolipoprotein components within the trypanosome lytic factor complex revealed in a novel transgenic mouse model. J Exp Med 2008; 205: 1721-1728.

14. Molina-Portela Medel, P, Lugli, EB, Recio-Pinto, E, Raper, J Trypanosome lytic factor, a subclass of high-density lipoprotein, forms cation-selective pores in membranes. Mol Biochem Parasitol 2005; 144: 218-226.

15. Rifkin, MR *Trypanosoma brucei*: Biochemical and morphological studies of cytotoxicity caused by normal human serum. Exp Parasitol 1984; 58:81-93.

16. Vanhollebeke, B, Lecordier, L, Perez-Morga, D, Amiguet-Vercher, A, Pays, E Human serum lyses *Trypanosoma brucei* by triggering uncontrolled swelling of the parasite lysosome. J. Eukaryot Microbiol 2007; 54: 448-451.

17. Raper, J, Fung R, Ghiso, J, Nussenzweig, V, Tomlinson, S Characterization of a novel trypanosome lytic factor in human serum. *Infect Immunity* 1999; 67: 1910-1916.

18. Vanhollebeke, B, Pays, E The trypanolytic factor of human serum; many ways to enter the parasite, a single way to kill. Mol Microb 2010; 76: 806-814.

19. DeGreef, C, Hamers, R The serum resistance-associated (SRA) gene of *Trypanosoma brucei rhodesiense* encodes a variant surface glycoprotein-like protein. Molec Biochem Parasitol 1994; 68: 277-284.

20. Turner, CMR, McLellan, S, Lindergard, LAG, Bisoni, L, Tait, A, MacLeod, A Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relation to sra expression. Parasitol 2004; 129: 445-454.

21. Xong, HV, Vanhamme, L, Chamekh, CE, Chimfwembe, Van Den Abbeele, J, Pays, A, Van Meirvenne, N, Hamers, R, De Baetselier, P, Pays, E A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. Cell 1998; 95: 839-846.

22. Stephens, NA, Hajduk, SL Endosomal Localization of the serum resistance-associated protein in African trypanosomes confers human infectivity. Eukaryot Cell 2011; 10: 1023-1033.

23. Kieft R, Capewell P, Turner CM, Veitch NJ, MacLeod A, Hajduk S Mechanism of *Trypanosoma brucei gambiense* (group 1) resistance to human trypanosome lytic factor. *Proc Natl Acad Sci U S A.* 2010; 107:16137-41.

24. Faulkner, SD, Oli, MW, Kieft, R, Cotlin L, Widener, J, Shiflett A, Cipriano, MJ, Pacocha, SE, Birkeland, SR, Hajduk, SL, McArthur, AG *In vitro* generation of human high density lipoprotein resistant *Trypanosoma brucei brucei*. Eukaryot Cell 2006; 5:1276-1286.

25. Green, HP, Pilar Molina Portela, M, St. Jean, EM, Lugli, EB, Raper J Evidence for a *Trypanosoma brucei* lipoprotein scavenger receptor. J Biol Chem 2003; 278: 422-427.

FIGURE LEGENDS

Figure 1. Characterization of purified TLF-1 and TLF-2. **(A)** Superose 6 size exclusion chromatography of TLF-1 and TLF-2. Absorbance profiles (280 nM) of TLF-1 and TLF-2, superimposed on individually ran marker proteins (1 = thyroglobulin (660kDa), 2 = apoferritin (480kDa), 3 = conalbumin (67kDa), 4 = ovalbumin (45kDa). **(B)** Analysis of individual Superose 6 column fractions of TLF-1 and TLF-2 separated on non-denaturing 12% SDS-PAGE and silver stained (top panel). Hpr, apoL1 and IgM were detected by western blot. (NA = indicates samples that were not analyzed)

Figure 2. *In vitro* activity of human serum, TLF-1 and TLF-2. TLF-1 resistant (R) and susceptible (S) clonal cell lines of bloodstream form *T. b. brucei* Lister 427 expressing VSG221, 800 and 060 were prepared as previously described[24,25]. The percentage surviving cells was determined, using phase contrast microscopy, 14 hours following the addition of TLF-1, TLF-2 or complete human serum to exponentially growing cultures at 37°C. **(A)** TLF-1 susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei* 427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green). **(B)** Normal human serum (NHS) susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei*

427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green). **(C)** TLF-2 susceptibility of *T. b. brucei* 427-221^{S} (black), *T. b. brucei* 427-800^{S} (blue), *T. b. brucei* 427-800^{R} (red), *T. b. brucei* 427-060^{S} (yellow) and 427-060^{R} (green).

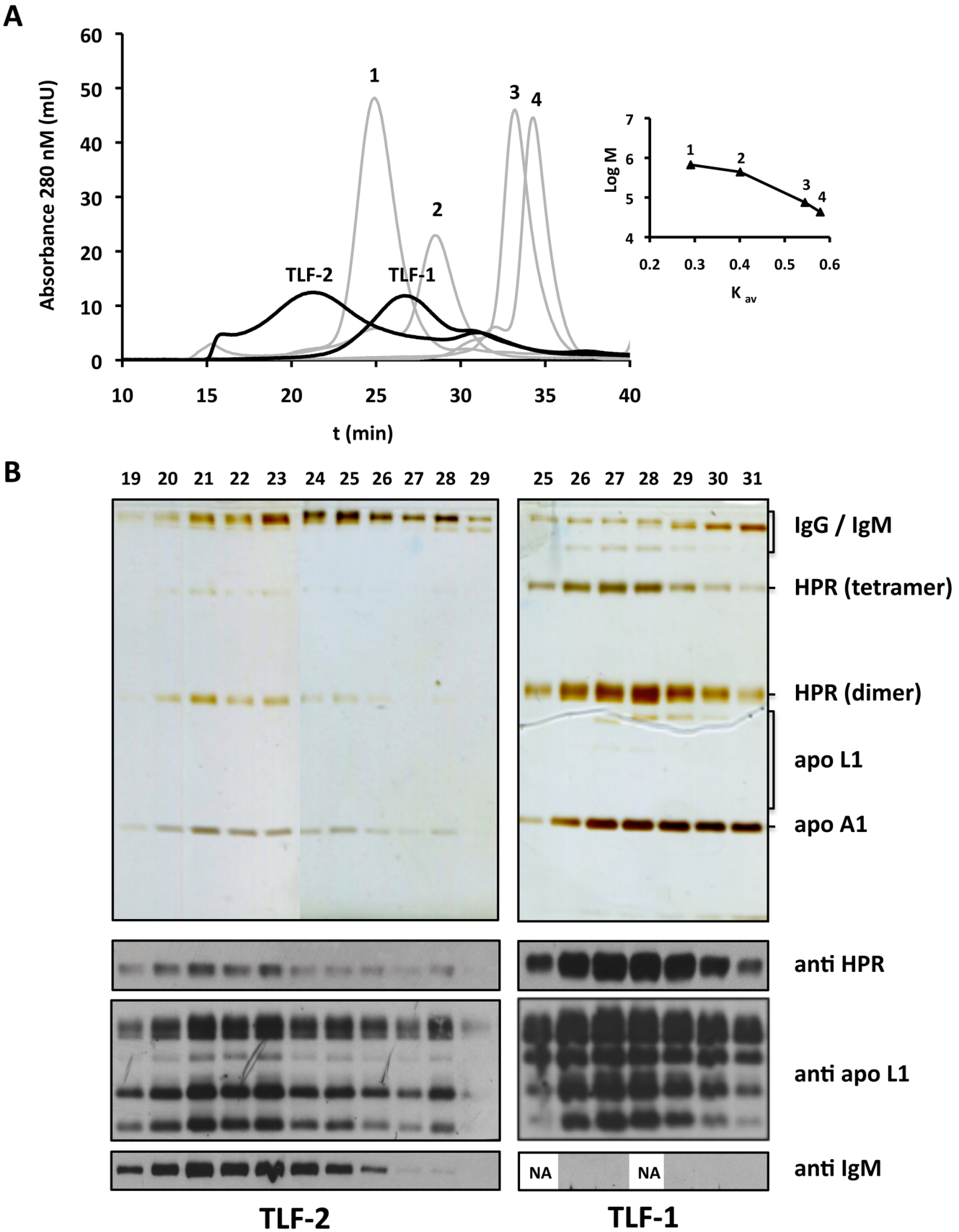

Figure 1

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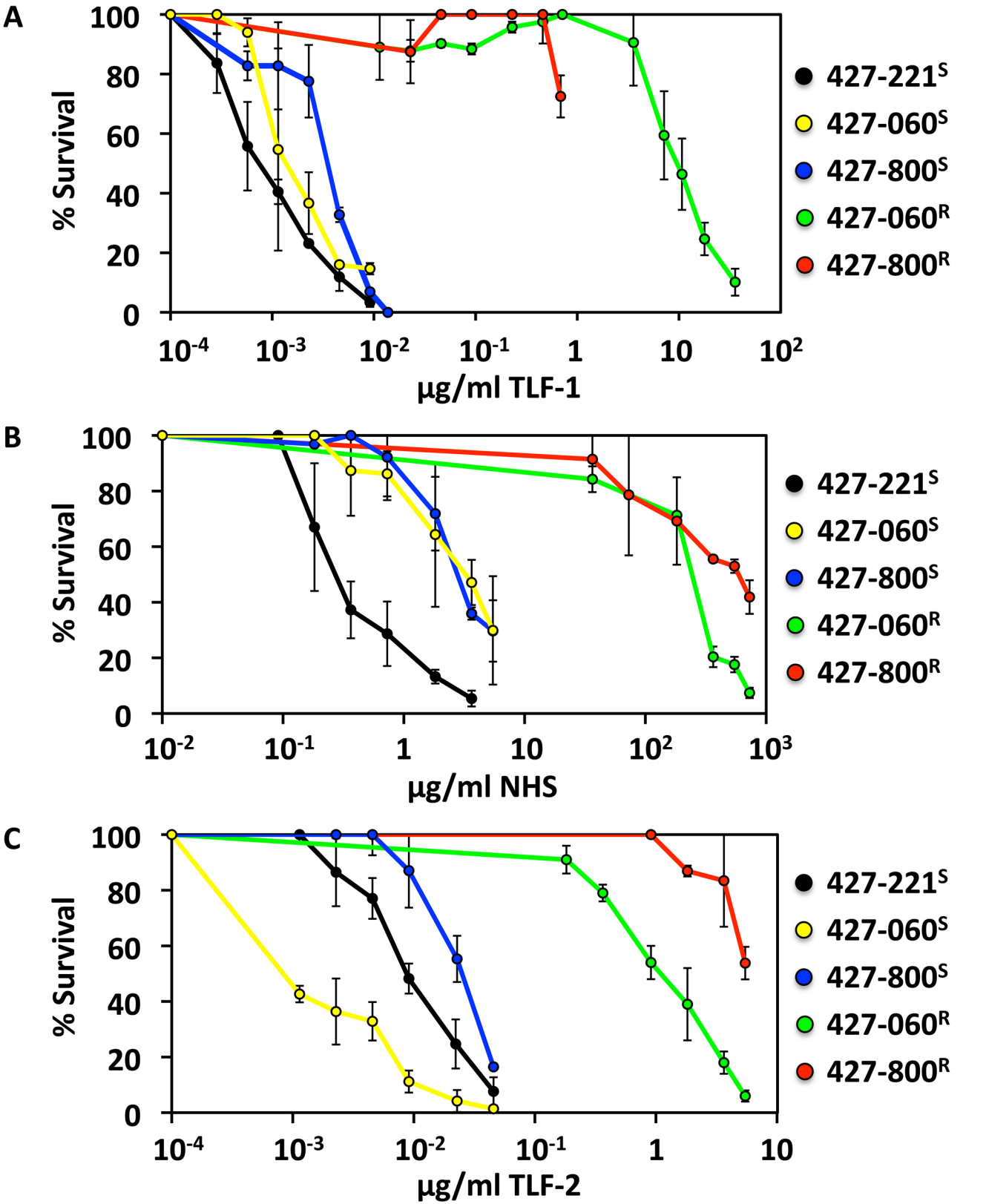

Figure 2

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